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ORIGINAL ARTICLE

Talcum powder induces malignant transformation in normal human primary ovarian epithelial cells

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ABSTRACT

BACKGROUND: Several studies have linked perineal use of talcum powder to increased risk of ovarian cancer (OC). Here, we determined that exposure to talcum powder induces malignant transformation in human normal ovarian cells.

METHODS: Human primary ovarian epithelial cells (HPOE), ovarian epithelial cells (HOSEpiC), and primary fibroblasts (NF) were treated with either 100 or 500 μg/mL of talcum powder or titanium dioxide (TiO_2) as a particulate control for 72 hours before assessment with a cell transformation assay and p53 and Ki-67 immunohistochemistry.

RESULTS: Treatment with talcum powder resulted in formation of colonies, indicating cell malignant transformation in a dose dependent manner in ovarian cell lines. No colonies formed in the untreated ovarian cells or control ovarian cells (TiO_2 treated) at either dose. There were no colonies formed in talc treated NF cells. Transformed ovarian cells were increased by 11% and 20% in HPOE and 24% and 40% in HOSEpic cells for talcum powder 100 and 500 μg/mL doses, respectively ($P<0.05$). There were no detectible transformed cells when cells were treated with TiO_2. Importantly, p53 mutant type as well as increased expression of Ki-67 were detected in HPOE and HOSEpic cells when exposed to talcum powder.

CONCLUSIONS: Exposure to talcum powder induces malignant transformation in ovarian epithelial cells but not in NF cells. These findings represent a direct effect of talcum powder exposure that is specific to normal ovarian cells and further supports previous studies demonstrating an association between the genital use of talcum powder and an increased risk of OC.

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KEY WORDS: Talc; Epithelial cells; Fibroblasts; Ovarian neoplasms; Cell proliferation.

Ovarian cancer is a gynecologic malignancy that ranks fifth in cancer deaths among women in USA.[1] Epithelial ovarian cancer (EOC) presents with various histopathology, molecular biology, and clinical outcome and is therefore considered a heterogeneous disease.[2] The pathogenesis of EOC is strongly associated with oxidative stress and inflammation.[3-5]

Epithelial ovarian cancer cells manifest a persistent pro-oxidant state that has been demonstrated *in vitro* and is also enhanced in chemoresistant EOC cells.[3, 4] Attenuation of the pro-oxidant state with antioxidants/scavengers has been shown in vitro to selectively induce apoptosis in EOC cells indicating a potential therapeutic value.[6, 7] Talcum Powder has been shown to induce oxidative stress and cell proliferation and to decrease apoptosis in normal ovarian cells and thus may play an important role in the pathogenesis of EOC.[8]

The association between genital use of talcum powder and risk of ovarian cancer have

been described in numerous studies.[8-11] Several meta-analyses have demonstrated a statistically significant increased risk of ovarian cancer with the genital use of talcum powder.[11-13] In addition, several animal studies have reported that talcum powder causes inflammation and oxidative stress.[14-16] Several *in-vitro* studies have demonstrated a biologic effect when cells in culture are exposed to talcum powder.[17-21]

In support of these previous findings, we have recently delineated the molecular basis of the association of talcum powder use with increased risk of ovarian cancer.[8] The specific mechanism by which talcum powder exposure causes ovarian cancer has not been definitively established. Here we clearly demonstrate that exposure to talcum powder induces malignant transformation in human primary normal ovarian epithelial cells and thus, providing a mechanism for the increased risk of ovarian cancer with the genital use of talcum powder.

Materials and methods

Normal human primary ovarian epithelial cells (HPOE)

Cells were received at passage 3 (Cell Biologics, Chicago, IL, USA) cryo-preserved in vials containing at least 0.5 $\times 10^6$ cells per mL. Cells were grown in gelatin pre-coated T25 flasks for 2 min and incubated in Cell Biologics Culture Complete Growth Medium. Cells were expanded for 2-4 passages at a split ratio of 1:2 under the cell culture conditions as specified by Cell Biologics. Human Epithelial Cell Medium is a complete medium designed for the culture of human epithelial cells. It was tested and optimized with epithelial cell growth and proliferation in vitro. Cells were incubated at 37 °C with 5% CO_2 and 95% air.

Normal human ovarian epithelial cells (HOSEpiC)

Cells were purchased from ScienCell Research Laboratories, Inc, Carlsbad, California. HOSEpiC cells were isolated from human ovary. HOSEpiC cells were received cryopreserved at passage one in frozen vials, each vial contains 5 $\times 10^5$ cells in 1 mL volume. Cells were further expanded for 2-3 passages in Ovarian Epithelial Cell Medium (OEpiCM, Cat. #7311). Cells were incubated at 37 °C with 5% CO_2 and 95% air.

Human normal primary peritoneal fibroblasts

This fibroblast cell line has been extensively characterized in previous studies.[22] Cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA), with 10% fetal bovine serum (FBS, Innovative Research, Novi, MI) and penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) as we have previously described.[22] Cells were incubated at 37 °C with 5% CO_2 and 95% air.

Talcum powder treatment

Talcum baby powder (Johnson & Johnson, New Brunswick, NJ, USA, #30027477, Lot#13717RA) or control particles, Titanium dioxide (TiO_2, Spectrum Chemical Corp, Lot No. 2EB0148) were used to treat cells. Talcum powder or TiO_2 were suspended in PBS (Stock solution of 50 mg/mL) and sonicated 3 times for 1 minute each with Sonic Dismembrator (Thermo Fisher Scientific, Model 100). Stock solutions were filtered through 30 µm nylon mesh filters. No visible loss of material has observed. Cells were seeded in 100 mm Petri cell culture dishes (1 $\times 10^6$) and were treated 24 hours later in duplicate in a fresh media with 100 or 500 µg/mL of talc or titanium dioxide (TiO_2) for 72 hours. Control: cells (30K) with media only and Negative control: cells (30K) with media and PBS. No cell death was observed after 72 hours in culture in control or treated cells. Titanium dioxide, a naturally occurring particle, has been classified in humans and animals as biologically inert.[19, 23] Titanium dioxide particles are produced and used as fine (~ 0.1-2.5 µm) and nanosize (<0.1 µm) particles.[23] In this study, we used TiO_2 as a particulate control to exclude the effect of material size. Culture plates were washed several times to remove residual particles and collected by trypsin in fresh media. Cells were counted and their concentration was adjusted with fresh media to 1.5 $\times 10^6$ cells/mL.

Cells were now ready to be assessed with cell transformation assay (colorimetric), according to the manufacturer protocol (Abcam-235698,

Cambridge, MA, USA). The 100 and 500 µg/mL doses were chosen based on our previous studies which showed talcum powder to induce changes in redox balance of cells at the molecular level.[8] The experiments were repeated 3 times with a fresh solution of talcum powder and TiO_2. This assay is more stable, faster and more sensitive than the traditional Soft-Agar Assay. Traditional assays require 3-4 weeks of incubation and inconsistent due to independent counting. An additional advantage of this assay is it's linear range from 10,000-400,000 cells.

A cell-dose curve was established as described in the manufacturer's protocol. Briefly, we used cells (5.34×10^5 cells/mL) were suspended in 1X DMEM/10% FBS medium. Cells were diluted into seven serial dilutions in a 1.5 mL centrifuge tubes. Serial dilution was performed using an 8-channel multi pipette by adding 150 µL of media to each well of a 96-well microplate. A 150 µL aliquot of the 5.34×10^5 cells/mL (80×10^3 cells) was added to the wells of the first duplicate row. A 150-µL aliquot from the first duplicate row was removed and added to the next well and mixed. The process was repeated until the seven serial dilutions were obtained. The final well was blank with media only and no cells. A 35-µL aliquot of 1X DMEM/10% FBS and 15 µL of WST working solution were then added into each well and incubate at 37 °C for 4 hours. The absorbance was measured by a microtiter plate reader at 450 nm (Figure 1).

Agarose and WST working solutions were prepared as described in the kit information sheet (Abcam-235698). The base agarose mix was added into the required wells in a 96-well plate and kept for 15 minutes at 4 °C to solidify the agarose. A top agarose layer stock solution was prepared by using talcum powder or TiO_2 treated stock cell solution of 1.5×10^6 cells/mL (30,000 cells per well, which is within the recommended range of the assay) in 1X DMEM/10% FBS medium. The agarose-cell mix was added into every well of a 96-well plate previously holding the solidified base agarose layer and placed at 4 °C for 10 minutes to solidify the layer. After placing the plate for 10 min at 37 °C, 1X DMEM/10% FBS medium was added to all the wells and incubated at 37 °C for 6-8 days. On the last day the upper medium on the top agarose layer was cautiously removed by pipetting. A 1X DMEM/10% FBS and WST working solution was added into each well, incubated for 4 hours at 37 °C. The absorbance was measured by a microtiter plate reader at 450 nm. Colonies of transformed cells were detected and photograph by a Zeiss Axiovert 40 C Inverted Phase Contrast Microscope with an Axio camera.

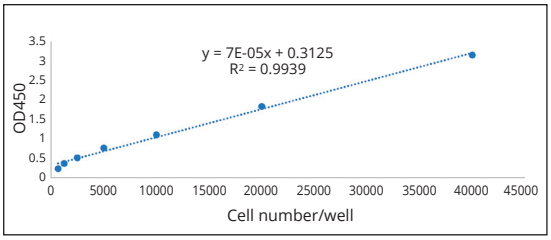

Figure 1.—Human primary normal ovarian epithelial cell-dose curve. The cell dose curve was established as described in methods using a serial dilution of cells.

Immunohistochemistry (IHC) staining and scoring

The IHC panel consisted of antibodies against p53 and Ki-67. The primary antibodies, suppliers, and staining conditions are listed in Table I.

Cytospin slides were prepared from cells and stained using immunoperoxidase labeling performed with the automated XT iVIEW DAB V.1 procedure on the Ultra BenchMark XT IHC/ISH Staining Module, Ventana with anti-p53 (clone DO-7 prediluted, Ventana). Antigen retrieval was carried out with CC1, pH 8.0 (Ventana). Sections were incubated with primary antibodies for 36 min at 37 °C. All slides were reviewed by two pathologists. Cases with discordant Ki-67 estimated results underwent a consensus review at a double-headed microscope. Diffuse "in-block" nuclear staining or complete negative staining with p53 was considered a positive reaction indicating mutated p53 status. Focal nuclear staining is consistent with "wild type" p53 and considered negative. The Proliferation

TABLE I.—*Primary antibodies, suppliers, and staining conditions.*

Antibody	Clone	Source	Detection System	Dilution
P53	DO-7	Ventana	Ventana ultraView DAB	1:500
Ki-67	Mib1	Ventana	Ventana ultraView DAB	1:2000

Index (PI) was assessed qualitatively using Ki-67-stained slides and classified as high PI (>50% positive cells) or low PI (<50% positive cell).

Statistical analysis

We performed ANOVAs with Tukey *post-hoc* tests to evaluate the difference between the three groups (no treatment control, talcum powder treatment and TiO_2 treatment). The values were expressed as mean and standard deviation. We used SPSS v. 24 for Windows (SPSS, Chicago, IL, USA); a $P<0.05$ defined significance.

Results

Treatment with talcum powder significantly increased the number of transformed normal epithelial ovarian cells by 11% and 20% in the 100 and 500 µg/mL talcum powder doses, respectively (Figure 2) ($P<0.05$). Likewise, but to a greater extent, treatment with talcum powder significantly increased the number of transformed HOSEpiC cells by 24% and 40% in the 100 and 500 µg/mL talcum powder doses, respectively (Figure 2) ($P<0.05$). Talcum powder had no detectable transformation effect on normal peritoneal fi-

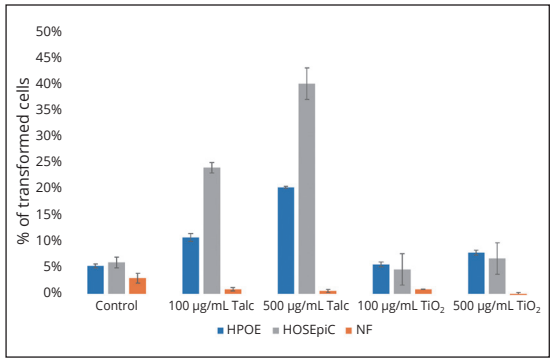

Figure 2.—Equal numbers (30K) of human primary normal ovarian epithelial cells (HPOE), human ovarian epithelial cells (HOSEpiC) and human normal peritoneal fibroblast cells (NF) were seeded for the cell transformation assay as described in methods. After 6 days, the cell number were measured. Standard and samples readings were taken 4 hours after adding WST working solution. Control: cells (30K) with media only.

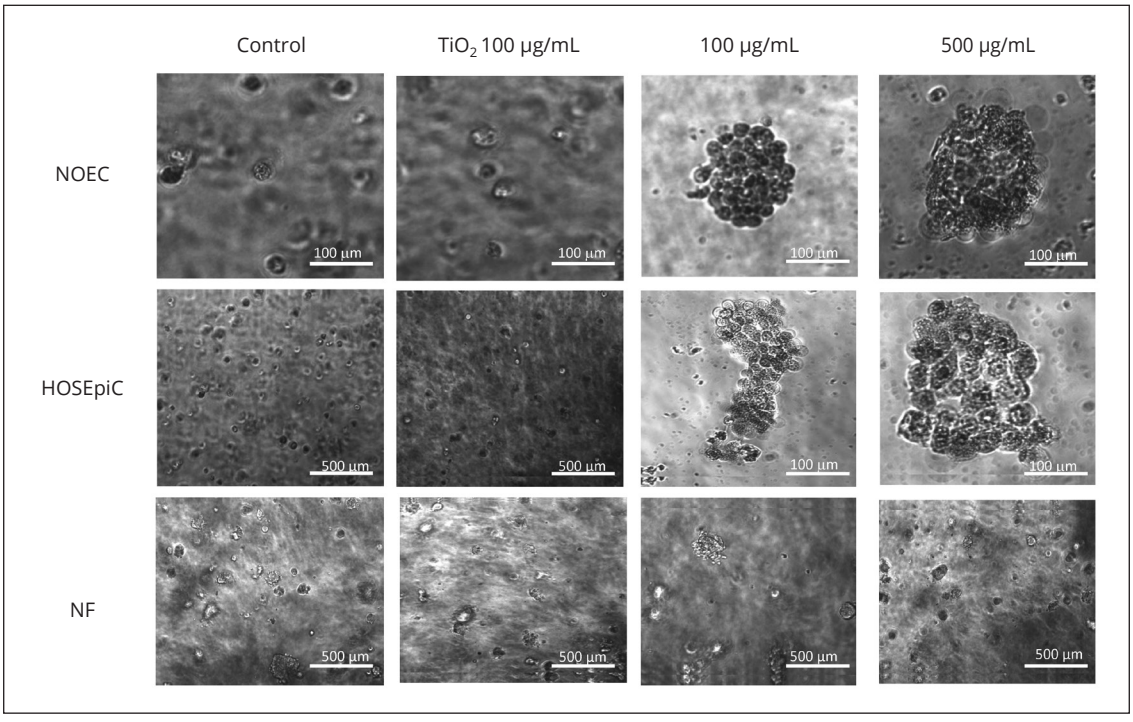

Figure 3.—Images of human primary normal ovarian epithelial cells (NOEC), human ovarian epithelial cells (HOSEpiC) and human normal peritoneal fibroblast cells (NF) treated with 100 and 500 µg/mL of talcum powder, after 6 days of culture. Colonies of transformed cells were detected and photograph by a Zeiss Axiovert 40 C Inverted Phase Contrast Microscope with an Axio camera.

broblasts at either dose (Figure 2). There was no significant difference between the no treatment control and the two doses of TiO_2 treatment control group (Figure 2) (P>0.05).

It is known that cancer cells are able to grow in culture without the need for matrix attachment. Treatment with talcum powder resulted in formation of colonies, indicating cell malignant transformation in normal epithelial ovarian cell lines in a dose dependent manner (Figure 3). There were no colonies formed in talcum powder treated normal fibroblasts (Figure 3). There were no colonies formed in either untreated ovarian cells or control ovarian cells at either dose. There were no detectible transformed cells when cells were treated with the particulate control, TiO_2.

To confirm malignant cell transformation observed with the cell transformation assay used in this study we performed IHC on the normal human primary ovarian epithelial (HPOE) and normal human ovarian epithelial cells (HOSEpiC) cells staining for p53 and Ki-67. Focal p53 nuclear staining indicating wild type p53 expression was observed in cells before treatment. After treatment of cells with talcum powder 100 µg/mL for 72 hours, diffused "in-block" nuclear staining was observed indicating p53 mutated form (Figure 4). Additionally, talcum powder treatment increased the proliferation index (PI) in both cell lines. The baseline PI for HPOE and HOSEpic cells was 50 and 70% respectively. The PI was significantly increased to 90% in both cell lines (Figure 4).

Discussion

This is the first study to directly show that exposure to talcum powder induces malignant transformation in ovarian epithelial cells. The ability of talcum powder exposure to induce transformation appears to be specific to ovarian cells as it did not induce transformation in peritoneal fibroblasts (Figure 3).

The link between talcum powder exposure and ovarian cancer have been supported by the harmful biological effects reported in various

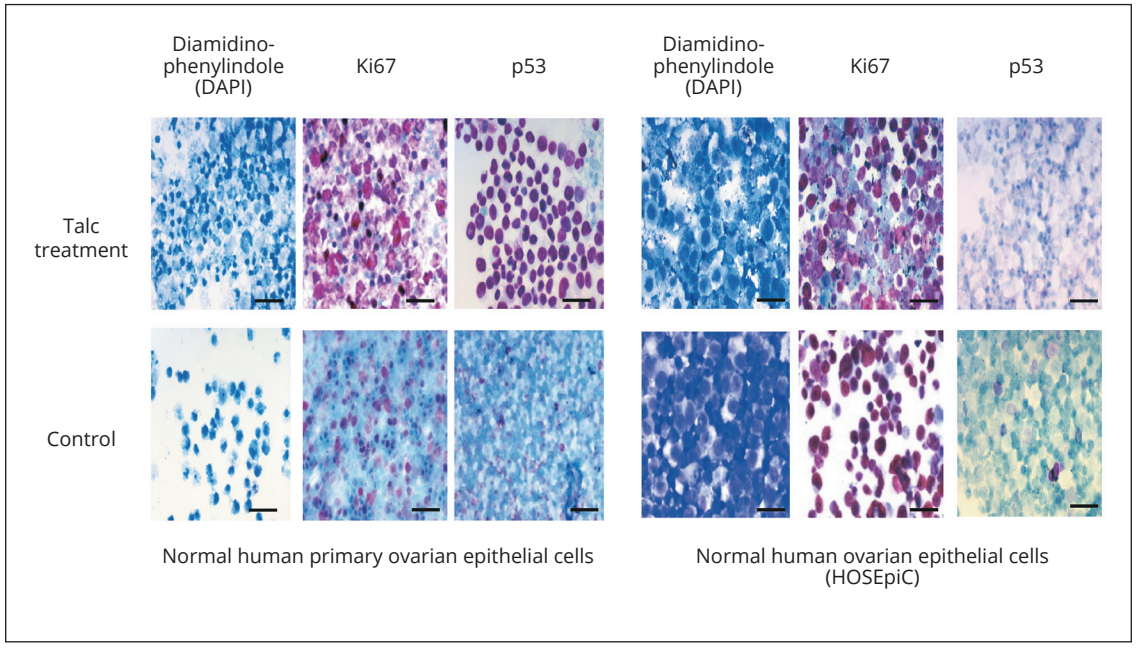

Figure 4.—Immunohistochemistry staining for p53 and Ki-67 in two normal human ovarian epithelial cells with and without Talcum powder (100 µg/mL) treatment for 72 hours. Slides were reviewed by two pathologists. Diffuse nuclear staining or complete negative staining with p53 is considered a positive reaction indicating mutated p53 status were observed in cells treated with talcum powder. Focal nuclear staining is consistent with wild type p53 and considered negative was observed in untreated cells (control). An increase in the proliferation index (Ki-67) was observes in talcum powder treated cells versus controls. Scale bar is 500 µm.

cell culture studies.[8, 14, 17-21, 24, 25] Macrophage activation and inflammatory response to talcum powder were suggested as a link to increased risk of ovarian cancer.[14, 18] Macrophages exposed to nano-talc manifested increased levels in inflammatory markers, TNF-alpha, IL-1beta and IL-6 as well as constituent phosphorylation of both p38 and ERK1/2 pathways.[18] p38 MAPK signaling pathway are known to be associated with cisplatin-resistant ovarian cancer.[26] Exposure of macrophages to talc and estradiol has led to increased production of reactive oxygen species and changes in expression of macrophage genes that play a role in cancer development and immunosurveillance.[24] These studies have also shown that ovarian cancer cells were present in larger numbers after co-culture with macrophages exposed to talc powder when in the presence of estradiol.[24]

Oxidative stress has been implicated in the pathogenesis of ovarian cancer, specifically, by increased expression of several key pro-oxidant enzymes in EOC tissues and cells as compared to normal cells.[3] Talcum powder exposure was shown to induce molecular changes in redox enzymes in normal ovarian cells similar to those known for ovarian cancer.[3, 8] In all talc-treated cells, there was a significant dose-dependent increase in key prooxidants with a concomitant decrease in key antioxidants enzymes. Remarkably, talcum powder exposure induced specific point mutations that are known to alter the activity in some of these key enzymes. The mechanism by which talcum powder alters the cellular redox and inflammatory balance involves the induction of specific mutations in key oxidant and antioxidant enzymes that correlate with alterations in their activities.[8]

We have previously identified a distinct oxidative stress/inflammation pattern for epithelial ovarian cancer and chemoresistant ovarian cancer.[3] We have utilized real time RT-PCR to measure levels of key redox and inflammation markers in response to talcum powder treatment and found that talcum powder altered specific markers to mimic the unique pattern characterized for ovarian cancer [8]. However, this effect was not observed in normal fibroblasts, hence, the effect is specific to epithelial ovarian cells.

Ovarian cancer cells were shown to manifest increased cell proliferation and decreased apoptosis, a hallmark of malignant cells, as compared to normal ovarian cells.[3] Indeed, talcum powder further enhanced cell proliferation and inhibited apoptosis in EOC cells, but more importantly in normal ovarian cells, suggesting talc is a stimulus to the development of the oncogenic phenotype.[8] Furthermore, CA-125, a membrane-bound and secreted protein, has been established as a biomarker for disease progression and response to ovarian cancer treatment.[27] CA-125 was significantly increased to values approaching clinical significance (35 U/mL in postmenopausal women) in talc treated normal ovarian cells.[8, 27] Thus, these findings confirmed the inflammatory/redox stress effects of talcum powder exposure to normal ovarian cells and indicated that this stress is a key mechanism in the malignant transformation of these cells.

The dose and time of talcum powder exposure in cell culture experiments used in this study was based on previous studies.[8] These doses are not intended to represent a typical dose when applied to the genital area in women over time. Despite this limitation, the development and use of in vitro models has been valuable in the advancement of research and knowledge on cancer pathogenesis.[28] The cellular transformation demonstrated in this study was significant and informative.

Anchorage-independent growth is one of the hallmarks of cell transformation and is accepted to be the most accurate and stringent in vitro assay for detecting malignant transformation of cells.[29, 30] The soft agar colony formation assay used in this study is widely accepted and used to evaluate cellular transformation.[29, 30] The Cell Transformation Assay Kit is faster, stable, more sensitive, and has a wide linear range (10,000-400,000) cells than the traditional Soft-Agar Assay. Therefore, in this study we used 30,000 of talcum powder and TiO_2 treated cells as well as control cells to stay within the recommended number of cells. The assay utilizes the conversion of tetrazolium salt to formazan by mitochondrial dehydrogenases which is directly proportional to the number of living transformed cells (Figure 2).

Tumor suppressor p53 gene mutations are frequently seen in ovarian cancers and can be used

as a biomarker to differentiate low from high grade serous ovarian carcinomas. The methods used for the assessment of p53 (mutant vs. wild type) and Ki-67 expression in this study is identical to the methods used in clinical pathology laboratories for the diagnosis of the different subtypes of ovarian cancer. The slides were scored and interpreted independently by two pathologists. Mutant p53 along with increased Ki-67 expression were detected in both HPOE and (HOSEpiC) ovarian cells treated with 100 μg/mL talcum powder for 72 hours (Figure 4). These findings supported the malignant transformation of normal ovarian cells seen in the agar transformation assay (Figure 3).

Conclusions

This study clearly demonstrate that talcum powder exposure induced malignant transformation of normal ovarian cells in culture which adds to the strong evidence of a causal relationship between the genital use of talcum powder and ovarian cancer. Therefore, we consider that future studies should aim to evaluate this finding utilizing animal models.

This study provides a step forward in understanding whether the distinct redox/inflammatory phenotype is the trigger of the oncogenic phenotype that leads to ovarian cancer. Indeed, we have shown that talcum powder in addition to altering the redox inflammatory status also alters p53 and Ki-67 genes, as in evidence of cell transformation. Cell transformation was also confirmed by the ability of talcum powder treated normal epithelial ovarian cells to form colonies using transformation assay. In conclusion, the ability of talcum powder exposure to induce malignant transformation appears to be specific to ovarian cells as it did not induce transformation in normal peritoneal fibroblasts. Further investigation to understand this specific effect of talcum powder on the ovaries is needed.

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Conflicts of interest.—Ghassan M. Saed has served as a paid consultant and expert witness for the plaintiffs in the talcum powder litigation. The remaining authors have no potential conflicts of interest to report.

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Authors' contributions.—Amy K. Harper performed and designed experiments, and helped writing manuscript. Xin Wang and Rong Fan performed the experiments. Thea Kirsch Mangu helped writing and revised manuscript. Nicole M. Fletcher designed the experiment, and helped writing the manuscript. Robert T. Morris interpreted the data and revised the manuscript. Ghassan M. Saed designed the experiments, supervised all aspects of this work, and wrote the manuscript. All authors read and approved the final version of the manuscript.

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